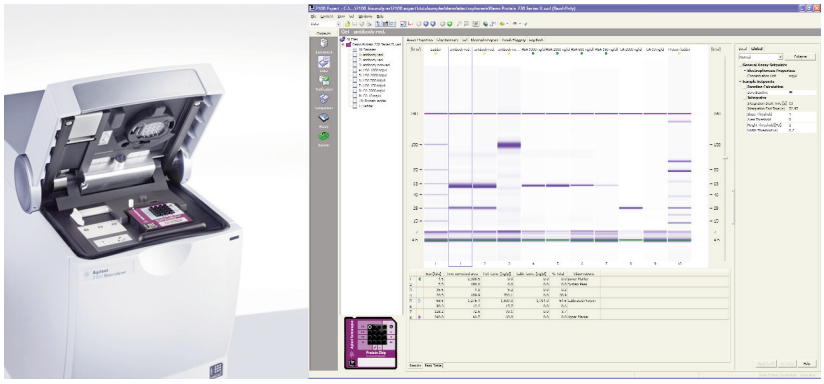
# Protein Sizing and Quantitation with the Agilent Protein 80 and Protein 230 Kits on the Agilent 2100 Bioanalyzer

Technical Overview



## Introduction

The Agilent 2100 Bioanalyzer employs microfluidic capillary gel electrophoresis for protein analysis where fluorescence intensities of proteins are measured as a function of their migration times. Data analysis is performed by the Agilent 2100 Expert Software, automatically determining molecular weight and concentration of the proteins in the sample. This Technical Overview describes the underlying mathematical operations for obtaining protein size and concentration information.

## Basic Principle of Protein Sizing and Quantitation

The two key parameters for protein sizing and quantitation with the Agilent 2100 Bioanalyzer are migration time and fluorescence intensity. The migration time relates to the size of the proteins: larger proteins move slower due to the sieving effect of the gel inside the chip channels. Fluorescence intensity, in turn, is related to the concentration of these proteins, which are indirectly stained by a fluorescent dye that intercalates with protein-SDS-micelles. This dye is part of the sieving gel matrix, which is put onto the chip and reacts with proteins during separation.

Since the migration time, as well as the fluorescence intensity, varies during the measurement of multiple samples, internal and external standards are used for calculating size and quantitation. The external standard is the specific protein ladder that is always analyzed on-chip before the actual sample measurements are done.

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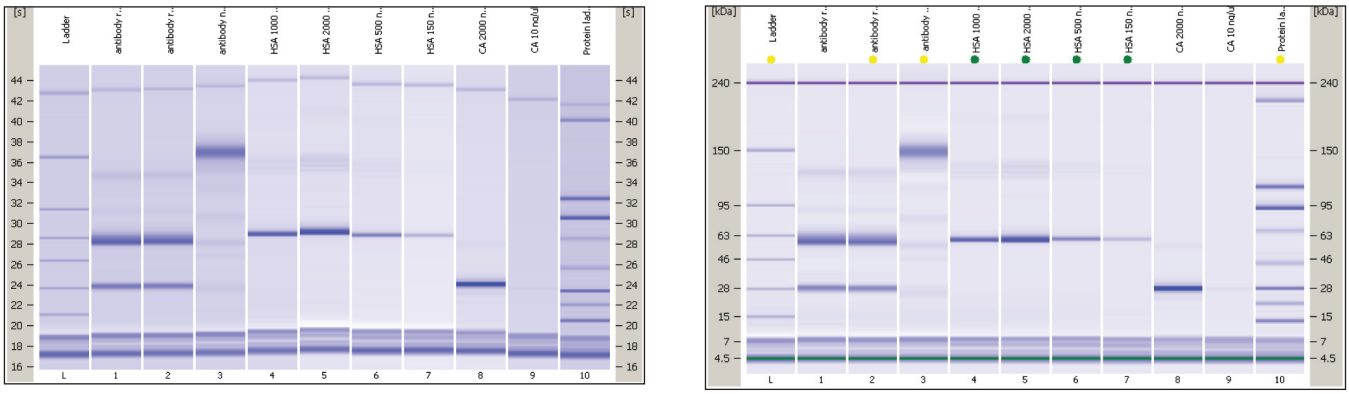

**Figure 1**
**Alignment of a P230 chip run. On the left the unaligned raw data is shown in the gel-like image representation. On the right, the same analysis after sample alignment based on lower marker (green) and upper marker (purple).**

Size and concentration are known for all ladder proteins. As an internal standard a protein of known size and concentration that is part of the sample buffer is added to each sample in a defined ratio, also called the upper marker. A lower marker is added to the sample as well. This marker is used for identifying and defining the measurement range and for alignment purposes. The lower marker is not a protein but rather a fluorescent dye. Sizing and quantitation algorithms are only part of a large number of data analysis steps. Before calculating the size and concentration, other steps have to be executed, like baseline corrections, specialized peak-finding algorithms and peak integration. Consequently there is no simple way to calculate sizing and quantitation information from raw data. In the following we describe some basic principles of the applied algorithms.

## Data Alignment

In theory, all upper markers and ladder peaks should always have the same migration time, since these proteins have the same size. However, similar to the smiling effect on SDS-PAGE gels, slight time shifts can occur for microfluidic assays due to changes in the electrophoretic boundary conditions over time.

Figure 1 shows this effect on the left side. The right side shows how the software has corrected for this effect by shifting the ladder and sample measurements to standardized values. As a result, all proteins having the same size and structure now also have the same corrected migration time.

## Protein Sizing

The migration time depends on the size of the proteins. Protein size determination is calculated via the standard curve from the ladder, the external standard of the Bioanalyzer protein kits.

In Figure 2, known ladder sizes are plotted against measured migration times. The blue point-to-point fit represents the standard curve, which is used to convert measured migration times of unknown proteins to their sizes. Since this size is determined by the external ladder standard, it can be compared across different measurements and different bioanalyzer instruments.

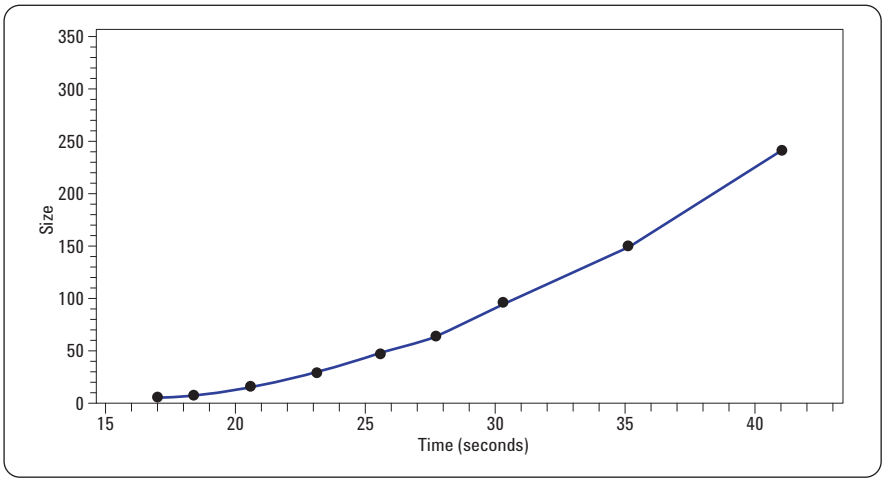


**Figure 2**
**Standard curve of the P230 ladder.**

## Relative Quantitation of Protein Concentration

The fluorescence intensity of a stained protein is proportional to its concentration. In order to calculate the concentration from the fluorescence intensity, two corrections need to be applied.

### Time correction

The Bioanalyzer detector measures the fluorescence intensity of moving molecules. Larger proteins migrating slower return a higher integral intensity, which is the peak area under the fluorescence curve, than smaller ones migrating faster through the detection path. In fact, the measured fluorescence is proportional to the reciprocal value of the migration time. Hence peak areas need to be corrected for this effect. The time-corrected peak area is calculated by dividing the raw peak areas by their corresponding migration times. Peak areas shown in the peak table represent time corrected areas.

### Normalization on upper marker

The upper marker is added to each sample in a defined ratio. Knowing the concentration of the upper marker $C_{UM}$ and measuring the peak areas for both the upper marker $A_{UM}$ and the protein peak $A_i$, allows for easy calculation of the protein concentration $C_i$ from the area ratios:

$$C_i = A_i \times (C_{UM}/A_{UM})$$

As $C_i$ is calculated relative to the upper marker concentration, it is also referred to as the relative concentration of the sample protein.

## Absolute Quantitation using Calibration Standards

Relative quantitation is based on the assumption that the ratio of concentration to peak area

$$C_{UM}/A_{UM} = C_i/A_i$$

is equal for all proteins and is not dependent on chemical properties of the fluorescent molecules. For example, this is true for the measurement of DNA samples, which always have a similar chemical composition and structure and therefore a similar staining efficiency. Conversely, different proteins can have a dissimilar staining efficiency. If the target protein has a different staining efficiency in comparison to the upper marker, a correction-factor *f* can be introduced:

$$C_{UM}/A_{UM} = f\,(C_i/A_i)$$

Correction-factor f can be determined by measuring the peak areas $A_i$ of calibration standards having known concentrations $C_i$. In a second step, this factor can be used to calculate the concentration of the target sample proteins having similar staining efficiency as the calibration standards.

For protein measurements with Agilent Protein 80 and Protein 230 kits on the Agilent 2100 Bioanalyzer, the calibration needs to be performed for each chip by analyzing a dilution series of the target protein. After measuring the calibration standards, a calibration curve is calculated converting the relative concentration determined with the upper maker to a calibrated or absolute concentration.

Figure 3 shows a calibration curve (blue line) derived from four data points that correlate the user input for known concentrations (calibration or "absolute" concentration) of an HSA standard to the relative concentration calculated with the upper marker as described earlier. The calibration curve (blue line) represents a least squares fit through the data points and is used for calculating the calibrated concentration for protein samples. The slope of this calibration curve, which is set to always intersect the origin, is the correction-factor *f* introduced above.

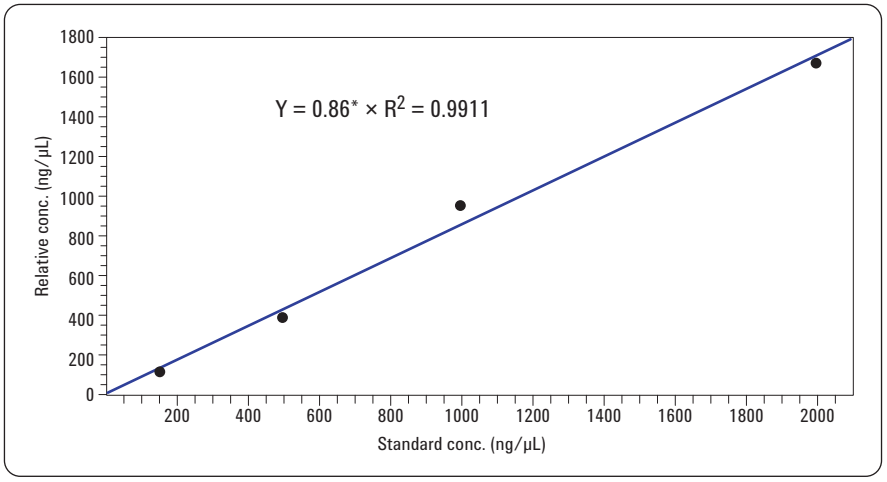


**Figure 3**
**Calibration curve derived from a dilution series of purified human serum albumin at four concentration points: 125, 500, 1000, and 2000 µg/mL .**

## Conclusion

Protein sizing and quantitation with the Agilent Protein 80 and Protein 230 kits for the Agilent 2100 Bioanalyzer are based on a number of mathematical calculations and algorithms. These are all performed automatically by the Agilent 2100 Expert Software. For size determination, peak migration times are compared to an external sizing standard; the ladder. For concentration determination, peak areas are compared to internal standard proteins; either the upper marker for relative quantitation or user-defined calibration standards for absolute quantiation.

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